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Novozymes A/S
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2880 Bagsvaerd
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Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
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TRANSMISSION OF THE CERTIFICATE FOR A EUROPEAN PATENT
PURSUANT TO RULE 54 (1) EPC

The certificate for a European patent, with the specification annexed thereto, is enclosed herewith.

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Urkunde Certificate Certificat

Es wird hiermit bescheinigt, daß für die in der beigefügten Patentschrift beschriebene Erfindung ein europäisches Patent für die in der Patentschrift bezeichneten Vertragsstaaten erteilt worden ist.

It is hereby certified that a European patent has been granted in respect of the invention described in the annexed patent specification for the Contracting States designated in the specification.

Il est certifié qu'un brevet européen a été délivré pour l'invention décrite dans le fascicule de brevet ci-joint, pour les Etats contractants désignés dans le fascicule de brevet.

Europäisches Patent Nr. **European Patent No.** **Brevet européen n°**

0784674

Patentinhaber **Proprietor of the Patent** **Titulaire du brevet**

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Munich,
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(56) References cited:
EP-A- 0 130 064
WO-A-94/03578
US-A- 5 439 811

- **DIALOG INFORMATION SERVICES, File 155, Medline, Dialog Accession No. 09225262, Medline Accession No. 95155262, NAGAO T. et al., "Cloning and Nucleotide Sequence of cDNA Encoding a Lipase from Fusarium Heterosporum"; & J. BIOCHEM., (TOKYO) (JAPAN), Sep. 1994, 116(3), p536-40.**
- **DIALOG INFORMATION SERVICES, File 5, BIOSIS, Dialog Accession No. 4087161, Biosis Accession No. 76037012, GUMENOV V.L. et al., "The Content of Cyclic Nucleotides and Phospho di Esterase Activity in the Ontogenesis of the Phyto Pathogenic Fungus Fusariumculmorum"; & PRIKL. BIOKHIM. MIKROBIOL., 18(5), 1982, 652-658.**
- **DIALOG INFORMATION SERVICES, File 5, BIOSIS, Dialog Accession No. 3130931, Biosis No. 70080838, LIN T.S. et al., "Isolation and Characterization of a Cuticular Poly Ester Cutin Hydrolyzing Enzyme from Phytopathogenic Fungi"; & PHYSIOL. PLANT. PATHOL., 17(1), 1980, 1-16.**
- **DIALOG INFORMATION SERVICES, File 155, Medline, Dialog Accession No. 03118570, Medline Accession No. 77020570, SOLIDAY C.L. et al., "Isolation and Characterization of a Cutinase from Fusarium Roseum Culmorum and Its Immunological Comparison With Cutinases from F. Solani Pisi"; & ARCH. BIOCHEM. BIOPHYS., (UNITED STATES), Sep. 1976, 176(1), p334-43.**

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Description

TECHNICAL FIELD

[0001] The present invention relates to novel lipolytic enzymes. More specifically the invention provides novel lipolytic enzymes derived from *Fusarium culmorum*.

BACKGROUND ART

[0002] Lipolytic enzymes find multiple industrial applications. Alkaline lipases are of particular interest for use in detergent compositions.

[0003] Alkaline lipases of microbial origin have been described, including lipases obtained from *Fusarium*. However, lipases obtained from *Fusarium culmorum* have never been disclosed.

SUMMARY OF THE INVENTION

[0004] It is an object of the present invention to provide novel alkaline lipolytic enzymes (EC 3.1.1.3).

[0005] Accordingly, in its first aspect, the invention provides a lipolytic enzyme derived from *Fusarium culmorum*.

[0006] The enzyme has a pH optimum in the range of from about 7 to about pH 9, more specifically around pH 8, when determined at 30°C with tributyrine as substrate.

[0007] The enzyme has the following N-terminal amino acid sequence (cf. SEQ ID NO:1):

Ala-Val-Ser-Val-Ser-Thr-Thr-Asp-Phe-Gly-Asn-Phe-Lys-Phe-Tyr-Ile-Gln-His-Gly-Ala-Ala-Ala-Tyr-Xaa-Asn-

[0008] In its second aspect, the invention provides a process for the preparation of the lipolytic enzyme, which process comprises cultivation of a lipase producing strain of *Fusarium culmorum* in a suitable nutrient medium, containing carbon and nitrogen sources and other inorganic salts, followed by recovery of the lipolytic enzyme.

[0009] In its third aspect, the invention provides a process for the preparation of the lipolytic enzyme, which process comprises isolating a DNA fragment encoding the lipolytic enzyme; combining the DNA fragment with an appropriate expression signal in an appropriate plasmid vector; introducing the plasmid vector into an appropriate host either as an autonomously replicating plasmid or integrated into the chromosome; cultivating the host organism under conditions leading to expression of the lipolytic enzyme; and recovering of the enzyme from the culture medium.

[0010] In further aspects, the invention provides detergent compositions, as well as a detergent additives, comprising the lipolytic enzyme of the invention.

[0011] Finally, the invention provides a biologically pure culture of the strain *Fusarium culmorum* CBS 513.94.

DETAILED DISCLOSURE OF THE INVENTION

The Microorganism

[0012] The invention provides lipolytic enzymes derived from a strain of the fungus *Fusarium culmorum*. *Fusarium culmorum* is a known species and strains of *Fusarium culmorum* have been deposited and are publicly available from depositary institutes, e.g. Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Germany, and American Type Culture Collection (ATCC), U.S.A.

[0013] In a preferred embodiment the invention provides a lipolytic enzyme derived from the strain *Fusarium culmorum* DSM 1094, *Fusarium culmorum* DSM *Fusarium culmorum* 62184, *Fusarium culmorum* DSM 62188, *Fusarium culmorum* DSM 62191, *Fusarium culmorum* DSM 62223, *Fusarium culmorum* ATCC 12656, *Fusarium culmorum* ATCC 15620, *Fusarium culmorum* ATCC 16430, *Fusarium culmorum* ATCC 16551, *Fusarium culmorum* ATCC 26556, *Fusarium culmorum* ATCC 34910, *Fusarium culmorum* ATCC 34913, *Fusarium culmorum* ATCC 36017, *Fusarium culmorum* ATCC 36879, *Fusarium culmorum* ATCC 36881, *Fusarium culmorum* ATCC 36886, *Fusarium culmorum* ATCC 44417, *Fusarium culmorum* ATCC 46040, *Fusarium culmorum* ATCC 56088, *Fusarium culmorum* ATCC 56089, *Fusarium culmorum* ATCC 60275, *Fusarium culmorum* ATCC 60362, *Fusarium culmorum* ATCC 62214, *Fusarium culmorum* ATCC 62215, *or Fusarium culmorum* ATCC 64075, or a mutant or a variant thereof.

[0014] In its most preferred embodiment the invention provides a lipolytic enzyme derived from the strain *Fusarium culmorum* CBS 513.94, or a muant or a variant thereof. This strain has been deposited according to the Budapest

Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at Centraalbureau Voor Schimmelcultures (CBS), Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, Netherlands, on 25 October 1994.

[0015] In another aspect, the invention provides a biologically pure culture of the strain *Fusarium culmorum* CBS 513.94.

Physico-Chemical Properties

[0016] In preferred embodiments, the lipolytic enzyme of the invention may be characterized by having one or more of the following physico-chemical properties.

[0017] The enzyme has a molecular weight of 28.4 kDa, as determined by mass spectrometry.

Preparation of the Lipolytic Enzyme

[0018] The lipolytic enzyme of the invention may be produced by cultivation of a strain of *Fusarium culmorum* in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the lipase. In a preferred embodiment, the lipase producing strain is the strain *Fusarium culmorum* CBS 513.94, or a mutant or a variant thereof.

[0019] The lipolytic enzyme may also be obtained by recombinant DNA-technology by methods known in the art *per se*, e.g. isolating a DNA fragment encoding the lipase, combining the DNA fragment with appropriate expression signal (s) in an appropriate vector, introducing the vector or parts thereof into an appropriate host, either as an autonomously replicating plasmid or integrated into the chromosome, cultivating the host organism under conditions leading to expression of the lipase, and recovering the lipase from the culture medium.

[0020] In preferred embodiments of the invention, the host organism is of bacterial origin, preferably a strain of *Escherichia coli*, or a strain of *Bacillus*, or a strain of *Streptomyces*, or of fungal origin, preferably a strain of *Aspergillus*, a strain of *Neurospora*, a strain of *Fusarium*, or a strain of *Trichoderma*, or a yeast cell, preferably a strain of *Saccharomyces*, or a strain of *Kluyveromyces*, or a strain of *Hansenula*, or a strain of *Pichia*.

[0021] After the cultivation, the lipolytic enzyme may be recovered and purified from the culture broth by conventional methods, such as hydrophobic chromatography, ion exchange chromatography or combinations thereof.

Lipolytic Activity

[0022] The lipolytic activity may be determined using tributyrine as substrate. This method is based on the hydrolysis of tributyrin by the enzyme, and the alkali consumption is registered as a function of time.

[0023] One Lipase Unit (LU) is defined as the amount of enzyme which, under standard conditions (i.e. at 30.0°C; pH 7.0; and tributyrine as substrate) liberates 1 µmol titratable butyric acid per minute. Gum Arabic is used as emulsifier.

[0024] A folder AF 95/5 describing this analytical method in more detail is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Detergent Compositions

[0025] The lipolytic enzyme of the invention may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly (ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

[0026] The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

[0027] The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzenesul-

fonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

[0028] The detergent composition may additionally comprise one or more other enzymes conventionally used in detergent compositions, such as an amylase, a cutinase, a protease, a cellulase, a peroxidiase, and/or an oxidase.

[0029] The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

[0030] The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

[0031] The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

[0032] The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

[0033] The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

[0034] The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

[0035] Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	7 - 12%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 4%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	14 - 20%
Soluble silicate (as $Na_2O,2SiO_2$)	2 - 6%
Zeolite (as $NaA1SiO_4$)	15 - 22%
Sodium sulfate (as Na_2SO_4)	0 - 6%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Sodium perborate (as $NaBO_3.H_2O$)	11 - 18%
TAED	2 - 6%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18})	1 - 3%

(continued)

Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	15 - 21%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	24 - 34%
Sodium sulfate (as Na_2SO_4)	4 - 10%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
Soap as fatty acid (e.g. C_{18-22} fatty acid)	1 - 3%
Sodium carbonate (as Na_2CO_3)	10 - 17%
Soluble silicate (as $Na_2O,2SiO_2$)	3 - 9%
Zeolite (as $NaA1SiO_4$)	23 - 33%
Sodium sulfate (as Na_2SO4)	0 - 4%
Sodium perborate (as $NaBO_3.H_2O$)	8 - 16%
TAED	2 - 8%
Phosphonate (e.g. EDTMPA)	0 - 1%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 12%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 5%
Zeolite (as $NaA1SiO_4$)	25 - 35%
Sodium sulfate (as Na_2SO_4)	0 - 10%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

5) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	12 - 18%
Soap as fatty acid (e.g. oleic acid)	3 - 13%
Alkenylsuccinic acid (C_{12-14})	0 - 13%
Aminoethanol	8 - 18%
Citric acid	2 - 8%
Phosphonate	0 - 3%
Polymers (e.g. PVP, PEG)	0 - 3%
Borate (as B_4O_7)	0 - 2%
Ethanol	0 - 3%
Propylene glycol	8 - 14%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

6) An aqueous-structured liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. oleic acid)	3 - 10%
Zeolite (as $NaA1SiO_4$)	14 - 22%
Potassium citrate	9 - 18%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. PEG, PVP)	0 - 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
Glycerol	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5 - 10%
Ethoxylated fatty acid monoethanolamide	3 - 9%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	5 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	20 - 40%
Sodium sulfate (as Na_2SO_4)	2 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	12 - 18%
TAED	2 - 7%

(continued)

Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0 - 5%

8) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 14%
Ethoxylated fatty acid monoethanolamide	5 - 11%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	4 - 10%
Soluble silicate (as $Na_2O.2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	30 - 50%
Sodium sulfate (as Na_2SO_4)	3 - 11%
Sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

9) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 12%
Nonionic surfactant	1 - 4%
Soap as fatty acid	2 - 6%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Zeolite (as $NaA1SiO_4$)	18 - 32%
Sodium sulfate (as Na_2SO_4)	5 - 20%
Sodium citrate (as $C_6H_5Na_3O_7$)	3 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	4 - 9%
Bleach activator (e.g. NOBS or TAED)	1 - 5%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. polycarboxylate or PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, perfume)	0 - 5%

10) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 23%
Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8 - 15%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. lauric acid)	0 - 3%
Aminoethanol	1 - 5%

(continued)

Sodium citrate	5 - 10%
Hydrotrope (e.g. sodium toluensulfonate)	2 - 6%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 1%
Ethanol	1 - 3%
Propylene glycol	2 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

11) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	20 - 32%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	6 - 12%
Aminoethanol	2 - 6%
Citric acid	8 - 14%
Borate (as B_4O_7)	1 - 3%
Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/ acrylic acid copolymer)	0 - 3%
Glycerol	3 - 8%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0 - 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzene-sulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25 - 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
Sodium carbonate (as Na_2CO_3)	8 - 25%
Soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
Sodium sulfate (as Na_2SO_4)	0 - 5%
Zeolite (as $NaA1SiO_4$)	15 - 28%
Sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
Bleach activator (TAED or NOBS)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	9 - 15%
Alcohol ethoxylate	3 - 6%

(continued)

Polyhydroxy alkyl fatty acid amide	1 - 5%
Zeolite (as $NaA1SiO_4$)	10 - 20%
Layered disilicate (e.g. SK56 from Hoechst)	10 - 20%
Sodium carbonate (as Na_2CO_3)	3 - 12%
Soluble silicate (as $Na_2O.2SiO_2$)	0 - 6%
Sodium citrate	4 - 8%
Sodium percarbonate	13 - 22%
TAED	3 - 8%
Polymers (e.g. polycarboxylates and PVP=	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0 - 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	4 - 8%
Alcohol ethoxylate	11 - 15%
Soap	1 - 4%
Zeolite MAP or zeolite A	35 - 45%
Sodium carbonate (as Na_2CO_3)	2 - 8%
Soluble silicate (as $Na_2O.2SiO_2$)	0 - 4%
Sodium percarbonate	13 - 22%
TAED	1 - 8%
Carboxymethyl cellulose	0 - 3%
Polymers (e.g. polycarboxylates and PVP)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

[0036] The lipolytic enzyme of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the lipase may be added in an amount corresponding to 0.001-100 mg of lipase per liter of wash liquor.

EXAMPLES

[0037] The invention is further illustrated with reference to the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

Example 1

Cultivation Example

[0038] Seed cultures of the strain *Fusarium culmorum* CBS 513.94 were produced in 500 ml shakeflasks containing 100 ml of the following composition :

Corn steep liquer (dried)	12 g/l
Glucose	24 g/l

[0039] To each flask is added 0.5 g $CaCO_3$ and 0.5 ml of oil.

[0040] pH is adjusted to 5.5 before autoclavation.

[0041] After 3 days at 26°C and 250 rpm. 5 ml of each of the seed cultures were inoculated in shakeflasks containing 100ml of the following medium:

Pepton, Difco 0118	6 g/l
Pepticase, Sheffield Products	4 g/l
Yeast extract, Difco 0127	3 g/l
Meat extract, Difco 0126	1.5 g/l
Dextrose, Roquette 101-0441	1 g/l
Olive oil, Sigma	10 g/l

[0042] pH is adjusted to 7.3-7.4 before autoclavation.

[0043] Cultivation took place for 9 days at 26°C and 250 rpm. The broths were centrifuged and the supernatants purified on a hydrophobic matrix (TSK gel Butyl-ToyoPearl 650 C column, available from Tosoh Corporation, Japan), and applied for further studies.

Example 2

Characterization Example

pH Optimum

[0044] The supernatant obtained according to Example 1 was subjected to the LU method for determining lipase activity described above, and the relation between pH and lipase activity of the lipolytic enzyme of the invention was determined at 30°C in the range of from pH 6 to pH 10.

[0045] The results of this characterization is presented in Fig. 1. The lipolytic enzyme has its pH optimum in the range of from about pH 7 to about pH 9, more specifically around pH 8.

Molecular Weight Determination

[0046] Mass spectrometry was done using matrix assisted laser desorption ionisation time-of flight (MALDI-TOF) mass spectrometry in a VG Analytical TofSpec. For mass spectrometry, 2 µl of sample obtained according to Example 1 were mixed with 2 µl saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 0.1% TFA:acetonitrile (70:30)), and 2 µl of the mixture spotted onto the target plate. Before introduction into the mass spectrometer the solvent was removed by evaporation. The sample was desorbed and ionised by 4 ns laser pulses (337 nm) at threshold laser power and accelerated into the field-free flight tube by an accelerating voltage of 25 kV. Ions were detected by a micro channel plate set at 1850 V. The spectra were calibrated externally with proteins of known mass.

[0047] A mass of 28.4 kDa was determined.

N-terminal Amino Acid Sequence

[0048] Using standard methods for obtaining and sequencing peptides [*Findlay & Geisow* (Eds.) (1989); Protein sequencing - a practical approach; IRL Press], the following 25 N-terminal amino acid residues of the lipolytic enzyme have been identified, as presented by SEQ ID NO:1 (where Xaa designates an unknown amino acid residue):

Ala-Val-Ser-Val-Ser-Thr-Thr-Asp-Phe-Gly-Asn-Phe-Lys-Phe-Tyr-Ile-Gln-His-Gly-Ala-Ala-Ala-Tyr-Xaa-Asn-

Example 3

Lipolytic Activity

[0049] Using a monolayer equipment (KSV-5000, KSV Instruments, Finland) it has been demonstrated that the lipolytic enzyme from *Fusarium culmorum* has considerably increased activity towards dicaprin in presence of long chained alcoholethoxylates.

[0050] A mixed monolayer in a well defined overall composition, made of a diglyceride substrate and a monocomponent alcoholethoxylate (AEO: Heptaethylene glycol monooctadecyl ether) is spread on an aqueous subphase (10 mM Glycine, pH 10.0, 0.1 mM EDTA, 25°C). The surface pressure is adjusted to the desired value, and a well-defined amount of enzyme (10 LU; lipase units as defined above) is injected into the subphase. Lipolytic action is manifested through the speed of a mobile barrier compressing the monolayer in order to maintain constant surface pressure as insoluble substrate molecules are hydrolysed into more water soluble reaction products. Using this assay, lipolytic enzymes are discriminated by a parameter β indicating the final area-fraction of substrate (dicaprin) left unhydrolysed by the enzyme as lipolytic activity stops.

[0051] In this way, the lipase of the invention was compared to an *Aspergillus* lipase conventionally used in detergents (Lipolase™, available from Novo Nordisk A/S, Denmark). The results are presented in Table 1, below.

Table 1.

Improved tolerance of lipolytic enzyme from *Fusarium culmorum* compared to Lipolase™.	
	β (30 mN/m)*
Lipolase™	57%
Fusarium culmorum lipase	25%

* Surface pressure employed.

[0052] These results show that when compared to Lipolase™, the lipolytic enzyme obtained from *Fusarium culmorum* is considerably more efficient when alcoholethoxylates are present in the substrate phase.

Example 4

Substrate Affinity

[0053] A procedure has been developed aiming at a simple comparison of the ability of lipolytic enzymes to accumulate on/in a substrate phase (olive oil) at alkaline pH (pH 9.0) and presence of the non-ionic surfactant Dobanol 25-7 (2500 ppm) in the aqueous phase.

Procedure

[0054]

1. Two identical buffer solutions (5 ml) are prepared in 20 ml sealable vials, ("Sample" (s) and "Reference" (r)).
2. Enzyme is added into "Sample" and "Reference" and the lipase concentration is determined (X LU/ml).
3. Olive oil is added onto the "Sample" and both lipase solutions are shaken vigorously. Incubation at 4°C over night.

4. Remaining lipase concentration in the aqueous phases is determined after incubation (Y_1 LU/ml; i=r,s).

Summary of incubation conditions

[0055]

Buffer	100 mM Glycine (5 ml).
pH	9.0.
Substrate	Olive oil (5 ml).
Temperature	4°C.
Lipase activity	5-10 LU/ml.
Incubation	Over night (24-26 hours).

Evaluation of data

[0056] The result is calculated by comparing the activity-loss upon incubation in the aqueous phase in contact with olive oil to the activity-loss in the aqueous phase in absence of olive oil:

$$\alpha = Y_s / Y_r \textit{ (see above)}$$

[0057] The results are presented in Table 2, below.

Table 2

Substrate Affinity	
Lipolytic Enzyme	**α (%)**
Lipolase™	99%
Fusarium culmorum lipase	99%

SEQUENCE LISTING

[0058]

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(A) ORGANISM: Fusarium culmorum
(B) STRAIN: CBS 513.94

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION:101..1433

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Val Ser Val Ser Thr Thr Asp Phe Gly Asn Phe Lys Phe Tyr Ile
1 5 10 15

Gln His Gly Ala Ala Ala Tyr Xaa Asn
20 25

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line s 9-14.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (*including postal code and country*)
Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Netherland

Date of deposit	Accession Number
25 October 1994	CBS 513.94

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet ☐

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

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Form PCT/RO/134 (July 1992)

Claims

1. A lipolytic enzyme, which:

   a) is derived from a strain of *Fusarium culmorum.*,
   b) has a pH optimum in the range of from about 7 to about pH 9, when determined at 30°C with tributyrine as substrate, and
   c) has the following N-terminal amino acid sequence:

   Ala-Val-Ser-Val-Ser-Thr-Thr-Asp-Phe-Gly-Asn-Phe-Lys-Phe-Tyr-Ile-Gln-His-Gly-Ala-Ala-Ala-Tyr-Xaa-Asn-

2. The lipolytic enzyme of claim 1, which is derived from the strain *Fusarium culmorum* CBS 513.94.

3. The lipolytic enzyme of claim 1 or 2, which has a molecular weight of 28.4 kDa by mass spectrometry.

4. A process for the preparation of a lipolytic enzyme of any of claims 1-3, which process comprises cultivation of a lipase producing strain of *Fusarium culmorum* in a suitable nutrient medium, containing carbon and nitrogen sources and other inorganic salts, followed by recovery of the lipolytic enzyme.

5. The process of claim 4, in which the lipase producing strain is the strain *Fusarium culmorum* CBS 513.94.

6. A process for the preparation of a lipolytic enzyme according to any of claims 1-3, which process comprises isolating a DNA fragment encoding the lipolytic enzyme; combining the DNA fragment with an appropriate expression signal in an appropriate plasmid vector; introducing the plasmid vector into an appropriate host either as an autonomously replicating plasmid or integrated into the chromosome; cultivating the host organism under conditions leading to expression of the lipolytic enzyme; and recovering of the enzyme from the culture medium.

7. The process of claim 6, in which the host organism is of bacterial origin, preferably a strain of *Escherichia coli*, or a strain of *Bacillus*, or a strain of *Streptomyces*, or of fungal origin, preferably a strain of *Aspergillus*, a strain of *Neurospora*, a strain of *Fusarium*; or a strain of *Trichoderma*, or a yeast cell, preferably a strain of *Saccharomyces*, or a strain of *Kluyveromyces*, or a strain of *Hansenula*, or a strain of *Pichia*.

8. A detergent composition comprising the lipolytic enzyme of any of claims 1-3.

9. The detergent composition of claim 8, which further comprises one or more other enzymes, in particular proteases, amylases, cellulases, oxidases, and/or peroxidases.

10. A detergent additive comprising the lipolytic enzyme of any of claims 1-3, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

11. A biologically pure culture of the strain *Fusarium culmorum* CBS 513.94.

Patentansprüche

1. Lipolytisches Enzym, welches:

   a) von einem *Fusarium culmorum* Stamm abstammt,
   b) ein pH-Optimum in dem Bereich von ungefähr 7 bis ungefähr pH 9 hat, wenn dieser bei 30°C mit Tributyrin als Substrat bestimmt wird, und
   c) die folgende N-terminale Aminosäuresequenz hat:

Ala-Val-Ser-Val-Ser-Thr-Thr-Asp-Phe-Gly-Asn-Phe-Lys-Phe-Tyr-Ile-Gln-His-Gly-Ala-Ala-Ala-Tyr-Xaa-Asn-

2. Lipolytisches Enzym nach Anspruch 1, welches von dem Stamm *Fusarium culmorum* CBS 513.94 abstammt.

3. Lipolytisches Enzym nach Anspruch 1 oder 2, welches ein Molekulargewicht von 28,4 kDa durch Massenspektrometrie hat.

4. Verfahren zur Zubereitung eines lipolytischen Enzyms nach einem der Ansprüche 1 bis 3, dieses Verfahren umfasst die Kultivierung eines Lipaseherstellenden Stammes von *Fusarium culmorum* in einem geeigneten Nährmedium, welches Kohlenstoff- und Stickstoffquellen und andere anorganische Salze enthält, gefolgt von der Gewinnung des lipolytischen Enzyms.

5. Verfahren nach Anspruch 4, in welchem der Lipase-herstellende Stamm der Stamm *Fusarium culmorum* CBS 513.94 ist.

6. Verfahren zur Zubereitung eines lipolytischen Enzyms nach einem der Ansprüche 1 bis 3, dieses Verfahren umfasst die Isolierung eines DNA-Fragments kodierend für das lipolytische Enzym; Kombinieren des DNA-Fragments mit einem geeigneten Expressionssignals in einem geeigneten Plasmidvektor; Einführen des Plasmidvektors in einen geeigneten Wirt entweder als ein sich autonom replizierendes Plasmid oder integriert in das Chromosom; Kultivieren des Wirtsorganismus unter Bedingungen, die zur Expression des lipolytischen Enzyms führen; und Gewinnung des Enzyms aus dem Kulturmedium.

7. Verfahren nach Anspruch 6, in welchem der Wirtsorganismus bakterieller Herkunft ist, bevorzugt ein *Escherichia coli* Stamm oder ein *Bacillus* Stamm oder ein *Streptomyces* Stamm oder fungaler Herkunft, bevorzugt ein *Aspergillus* Stamm, ein *Neurospora* Stamm, ein *Fusarium* Stamm oder ein *Trichoderma* Stamm oder eine Hefezelle, bevorzugt ein *Saccharomyces* Stamm oder ein *Kluyveromyces* Stamm oder ein *Hansenula* Stamm oder ein *Pichia* Stamm.

8. Detergens-Zusammensetzung umfassend das lipolytische Enzym nach einem der Ansprüche 1 bis 3.

9. Detergens-Zusammensetzung nach Anspruch 8, welche ferner ein oder mehrere andere Enzyme umfasst, insbesondere Proteasen, Amylasen, Cellulasen, Oxidasen und/oder Peroxidasen.

10. Detergens-Zusatz umfassend das lipolytische Enzym nach einem der Ansprüche 1 bis 3, bereitgestellt in Form eines Granulats, vorzugsweise eines nichtstaubenden Granulats, einer Flüssigkeit, insbesondere eine stabilisierte Flüssigkeit, eines Schlamms oder eines geschützten Enzyms.

11. Biologisch reine Kultur des Stammes *Fusarium culmorum* CBS 513.94.

Revendications

1. Enzyme lipolytique, qui :

   a) est dérivée d'une souche de *Fusarium culmorum*,
   b) a un optimum de pH dans le domaine d'environ 7 à environ pH 9, quand il est déterminé à 30°C avec la tributyrine comme substrat, et
   c) a la séquence d'acides aminés N-terminale suivante :

Ala-Val-Ser-Val-Ser-Thr-Thr-Asp-Phe-Gly-Asn-Phe-Lys-Phe-Tyr-Ile-Gln-His-Gly-Ala-Ala-Ala-Tyr-Xaa-Asn-.

2. Enzyme lipolytique selon la revendication 1 qui est dérivée de la souche *Fusarium culmorum* CBS 513.94.

3. Enzyme lipolytique selon la revendication 1 ou 2 qui a une masse moléculaire de 28,4 kDa par spectrométrie de masse.

4. Procédé de préparation d'une enzyme lipolytique selon l'une quelconque des revendications 1-3, lequel procédé comprend la culture d'une souche de *Fusarium culmorum* productrice de lipase dans un milieu nutritif approprié, contenant des sources de carbone et d'azote et d'autres sels inorganiques, puis la récupération de l'enzyme lipolytique.

5. Procédé selon la revendication 4 où la souche productrice de lipase est la souche *Fusarium culmorum* CBS 513.94.

6. Procédé de préparation d'une enzyme lipolytique selon l'une quelconque des revendications 1-3, lequel procédé comprend l'isolement d'un fragment d'ADN codant l'enzyme lipolytique, la combinaison du fragment d'ADN avec un signal d'expression approprié dans un vecteur plasmidique approprié, l'introduction du vecteur plasmidique dans un hôte approprié, sous forme de plasmide à réplication autonome ou sous forme intégrée dans le chromosome, la culture de l'organisme hôte dans des conditions conduisant à l'expression de l'enzyme lipolytique, et la récupération de l'enzyme à partir du milieu de culture.

7. Procédé selon la revendication 6 où l'organisme hôte est d'origine bactérienne, de préférence une souche de *Escherichia coli*, ou une souche de *Bacillus*, ou une souche de *Streptomyces*, ou d'origine fongique, de préférence une souche de *Aspergillus*, une souche de *Neurospora*, une souche de *Fusarium*, ou une souche de *Trichoderma*, ou une cellule de levure, de préférence une souche de *Saccharomyces*, ou une souche de *Kluyveromyces*, ou une souche de *Hansenula* ou une souche de *Pichia*.

8. Composition détergente comprenant l'enzyme lipolytique selon l'une quelconque des revendications 1-3.

9. Composition détergente selon la revendication 8 qui comprend en outre une ou plusieurs autres enzymes, en particulier des protéases, des amylases, des cellulases, des oxydases et/ou des peroxydases.

10. Additif de détergent comprenant l'enzyme lipolytique selon l'une quelconque des revendications 1-3, fournie sous forme de granulés, de préférence de granulés ne produisant pas de poussière, d'un liquide, en particulier d'un liquide stabilisé, d'une suspension ou d'une enzyme protégée.

11. Culture biologiquement pure de la souche *Fusarium culmorum* CBS 513.94.

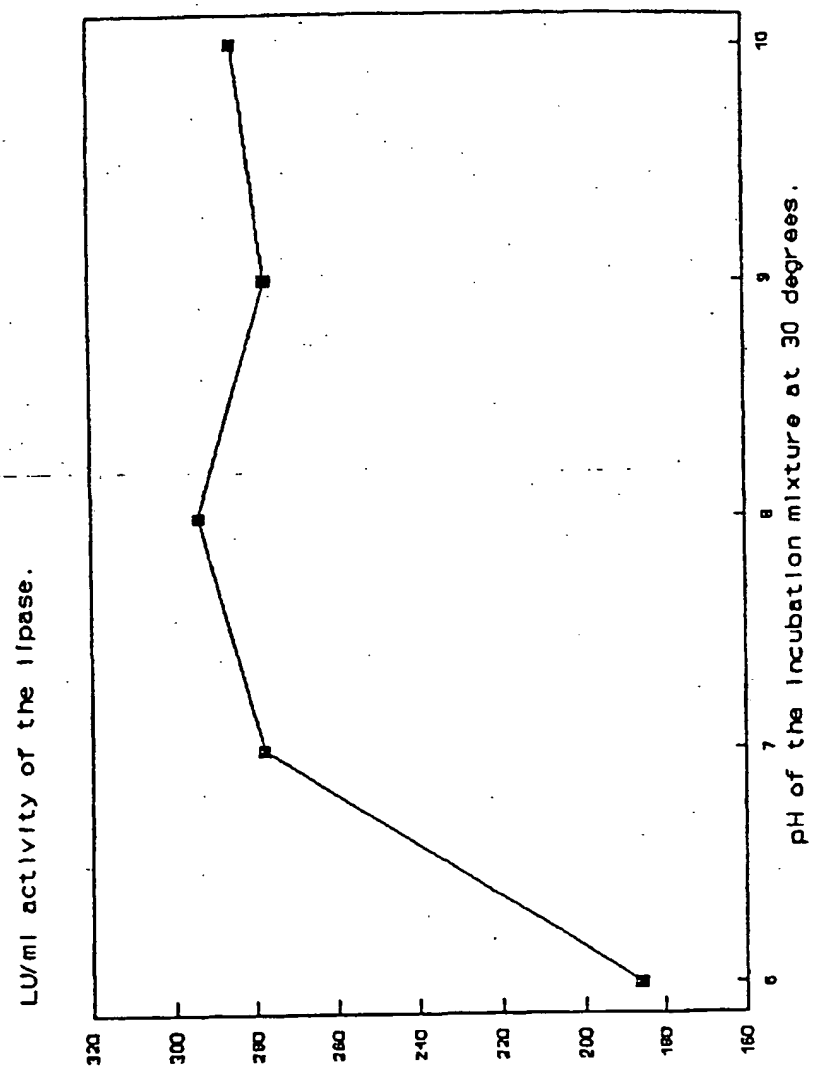

FIG. 1